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FULL PAPER

Evaluation of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2}:342}$ Affibody® molecule for *in vivo* molecular imaging

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Objective: The aim of this study was to develop an improved method for labelling $\text{Z}_{\text{HER2}:342}$ with Technetium-99m (^{99m}Tc) using Gly-(D) Ala-Gly-Gly as a chelator and to evaluate the feasibility of its use for visualization of HER2 expression *in vivo*.

Methods: The Affibody® molecule $\text{Z}_{\text{HER2}:342}$ was synthesized by Fmoc/tBu solid phase synthesis. The chelator, Gly-(D) Ala-Gly-Gly, was introduced by manual synthesis as the *N*-terminal extensions of $\text{Z}_{\text{HER2}:342}$. $\text{Z}_{\text{HER2}:342}$ was labelled with ^{99m}Tc . The labelling efficiency, radiochemical purity and *in vitro* stability of the labelled molecular probe were analysed by reversed-phase high performance liquid chromatography. Biodistribution and molecular imaging using ^{99m}Tc -peptide- $\text{Z}_{\text{HER2}:342}$ were performed.

Results: The molecular probe was successfully synthesized and labelled with ^{99m}Tc with the labelling efficiency of $98.10 \pm 1.73\%$ ($n = 5$). The radiolabelled molecular

probe remained highly stable *in vitro*. The molecular imaging showed high uptake in HER2-expressing SKOV-3 xenografts, whereas the MDA-MB-231 xenografts with low HER2 expression were not clearly imaged at any time after the injection of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2}:342}$. The predominant clearance pathway for ^{99m}Tc -peptide- $\text{Z}_{\text{HER2}:342}$ was through the kidneys.

Conclusion: ^{99m}Tc -peptide- $\text{Z}_{\text{HER2}:342}$ using Gly-(D) Ala-Gly-Gly as a chelator is a promising tracer agent with favourable biodistribution and imaging properties that may be developed as a radiopharmaceutical for the detection of HER2-positive malignant tumours.

Advances in knowledge: The ^{99m}Tc -peptide- $\text{Z}_{\text{HER2}:342}$ molecular probe is a promising tracer agent, and the results in this study provide a foundation for future development of protocols for earlier visual detection of cancer in the clinical setting.

The human epidermal growth factor receptor Type 2 (ErbB2), also known as HER2 or p185, is a trans-membrane tyrosine kinase receptor. HER2 overexpression has been detected in a number of malignant tumours, such as carcinomas of the breast, ovary and prostate.¹ Blocking of HER2 signalling using the monoclonal antibody trastuzumab (Herceptin) can improve the survival of patients with HER2-positive cancer.² As not all tumours express HER2, an accurate method for detection of this marker is required to select patients who can benefit from trastuzumab therapy.

Currently, the most widely used methods for evaluating receptor expression on tumours and metastases are immunohistochemical staining and fluorescent *in situ* hybridization of biopsy samples.³ However, the deficiencies in using biopsies are false-negative findings due to sampling errors and discordance in HER2 expression between

the primary tumour and metastases. Moreover, it is not possible to biopsy tumours at all sites. Targeted radionuclide imaging may help to avoid such issues by visualizing HER2 expression in both primary tumours and metastases. Meanwhile, compared with traditional imaging techniques, such as MRI, CT and ultrasound imaging, radionuclide imaging is attractive because it specifically detects expression of tumour markers such as HER2 rather than gross anatomical changes.

Monoclonal antibodies have often been used to target HER2 for radionuclide imaging, but slow uptake in tumours and slow blood clearance are well-recognized problems of these agents.^{4–6} Reduction of tracer molecular size is considered a promising way to improve imaging contrast by increasing the rates of tumour localization and clearance from blood and healthy tissues.⁷ The Affibody® molecule $\text{Z}_{\text{HER2}:342}$ is a 58-amino acid 3-helix bundle protein that originates from

the B-domain of the staphylococcal protein with a low molecular weight of about 7 kDa. Z_{HER2:342} has been reported to bind to the extracellular domain of HER2 with an affinity of 22 pM.⁸ Z_{HER2:342}, labelled with ¹²⁵I and ¹¹¹In, can target HER2-expressing xenografts with high specificity.^{9,10} The labelling of this protein with ⁶⁸Ga has also provided high-quality imaging of HER2-expressing tumours in patients.¹¹ In addition, radionuclides such as ¹⁸F, ¹⁸⁶Re, ¹⁷⁷Lu and Technetium-99m (^{99m}Tc) have been used to label Affibody molecules.^{12–14} Of note, HER2-binding Affibody molecules have been successfully developed and studied in conjunction with various radiolabels for diagnostic imaging applications. However, an optimal radiotracer is still not available.

The aim of the study was to develop an improved method for labelling Z_{HER2:342} with ^{99m}Tc using Gly-(D) Ala-Gly-Gly as a chelator and to evaluate the feasibility of its use in the visualizing of HER2 expression *in vivo*.

METHODS AND MATERIALS

Peptide synthesis and characterization

The Affibody molecule Z_{HER2:342} (VENKFNKEMRNAYWEIALPNNQQKRAFIRSLYDDPSQSANLLAEAKKLNDQAQPK) was synthesized by Shanghai Science Peptide Biological Technology Co., Ltd, Shanghai, China. Fmoc/tBu solid phase synthesis was used on a peptide synthesizer with a substitution of 0.67% mmol g⁻¹. 10 molar equivalents of Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, were used to activate equal molar equivalents of Fmoc-protected amino acid, and acetic anhydride was used to terminate peptides where acylation was incomplete. The Tc-chelating moieties were introduced to the N-terminal extensions of Z_{HER2:342} by manual synthesis. The N-terminal Fmoc protecting group was removed by incubation with 20% piperidine-*N*-methylpyrrolidone for 20 min. Peptides were released from solid support and deprotected by a 2-h incubation in trifluoroacetic acid (TFA):ethanedithiol:H₂O:triisopropylsilane (94.0:2.5:2.5:1.0) followed by extraction with tert-butyl methyl ether:H₂O (50:50) three times before filtration and lyophilization. At the N-terminus of the Z_{HER2:342} sequence, four amino acids (Gly-(D) Ala-Gly-Gly), forming an N₄ configuration were used as a linker for coupling Z_{HER2:342} and ^{99m}Tc.¹⁵ In addition, one γ -aminobutyric acid (γ -Aba) was introduced as a barrier to prevent steric hindrance,

and then Z_{HER2:342} was labelled with ^{99m}Tc by the ligand exchange method as shown in Figure 1. To verify the identity of the peptide, reversed-phase high performance liquid chromatography (RP-HPLC) was performed. In addition, mass spectrometric analysis was carried out on a mass spectrometer (LCMS-2011; Shimadzu Corp., Kyoto, Japan) with an electrospray ionization source to confirm the protein mass.

Radiolabelling with ^{99m}Tc

To obtain ^{99m}Tc-pertechnetate, the ⁹⁹Mo-⁹⁹Tc generator (Beijing Atom HighTech Co., Ltd., Beijing, China) was eluted with sterile 0.9% sodium chloride. Before labelling, freeze-dried Z_{HER2:342} was dissolved in distilled water and stored at -20 °C. For labelling, 20 μ L of Z_{HER2:342} (1 mg mL⁻¹) was mixed with 20 μ L of 0.15 M NaOH to obtain the final pH of about 11. Thereafter, 35 μ L of a solution of SnCl₂·2H₂O in 0.01 M HCl (1 mg mL⁻¹) was added, followed by 600 μ L (370 MBq) of fresh ^{99m}Tc-pertechnetate solution. The mixture was lightly vortexed and incubated for 60 min at room temperature. The labelling efficiency and radiochemical purity of the labelled conjugate were analysed using RP-HPLC (Alltech 305, Deerfield, IL) using a 4.6 $\times$ 250 mm C18 column with a particle size of 5 μ m, a 30-min gradient of 5–70% A (A: 0.1% TFA-CH₃CN; B: 0.1% TFA-H₂O) and a flow rate of 1 mL min⁻¹.

In vitro stability analysis

To assess the stability of the radiotracer *in vitro*, ^{99m}Tc-peptide-Z_{HER2:342} was incubated in saline or fresh human serum at 37 °C, and the stability of the radiotracer *in vitro* was evaluated at 1, 2, 4, 6 and 24 h by RP-HPLC using the same conditions as those for testing labelling efficiency above.

Cell culture

The HER2-expressing ovarian carcinoma cell line, SKOV-3 (displaying approximately 1.2 $\times$ 10⁶ HER2 receptors per cell),¹⁶ and human breast carcinoma cell line, MDA-MB-231 with low HER2 expression (4 $\times$ 10⁴ HER2 receptors per cell),¹⁷ were purchased from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultivated in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA) under standard conditions (37 °C, humidified atmosphere containing 5% CO₂). Cell growth was

Figure 1. Radiolabelling of peptide-Z_{HER2:342} with Technetium-99m (^{99m}Tc).

monitored under an inverted microscope with phase contrast. When the cell density reached 90%, SKOV-3 and MDA-MB-231 cells were harvested using trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; 0.25% trypsin, 0.02% EDTA; Invitrogen).

Establishment of xenograft tumours in nude mice

All animal studies were approved by the Animal Care and Use Committee of Hebei Medical University. BALB/c nude mice (females; weight, 20 ± 4 g; 4–6 weeks old) bearing HER2-expressing SKOV-3 cells or MDA-MB-231 cells with low HER2 expression were used in the targeting experiments. The animals were purchased from the Department of Laboratory Animal Science of Peking University Health Science Centre, Beijing, China, and acclimated for 7 days in the Department of Laboratory Animal Science (the Fourth Hospital of Hebei Medical University, Hebei, China) before tumour implantation. A total of 5×10^6 SKOV-3 or MDA-MB-231 cells in 200 μ l of RPMI 1640 medium without phosphate buffered saline were injected subcutaneously in the right upper limbs of each mouse. The tumours were allowed to grow to a diameter of 1–1.5 cm for about 4–6 weeks.

Biodistribution studies

20 SKOV-3 bearing BALB/c nude mice were randomly divided into 4 groups with 5 mice in each group. Labelled conjugates (1110 kBq) were injected into each mouse via the tail vein. At 1, 4, 6 and 8 h after injection, the mice were sacrificed by cervical dislocation. The organ or tissue samples of interest (blood, heart, liver, spleen, kidney, lung, stomach, small intestine, brain, bone, muscle and tumour) were harvested by dissection and weighed. A gamma well counter (CAPRAC®; Capintec Inc., Ramsey, NJ) was used to measure radioactivity uptake in organs or tissues, which was calculated as the percentage of injected dose per gram of tissue (%ID/g). Meanwhile, ratios of radioactivity in the tumour to that in blood, liver and muscle were also calculated to evaluate tumour uptake in this study. In addition, the low HER2-expressing MDA-MB-231 cell line was used as a “negative control” to evaluate the specificity of ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ uptake in tumours. 20 BALB/c mice bearing low HER2-expressing MDA-MB-231 xenografts were used in the biodistribution studies, with 5 mice in each group. These animals were injected with the same dose of ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$, sacrificed at the same time points and processed in the same manner as described above for the SKOV-3 xenograft model. To further analyse the binding specificity of ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ to HER2-expressing cells, 5 mice were intravenously injected with 500 μ g of unlabelled $Z_{\text{HER2:342}}$ 1 h before the labelled conjugates were injected to saturate HER2 receptors. At 4 h after injection of the labelled molecular probe, all five mice were sacrificed, and organs and tissue of interest were collected, weighed and analysed for radioactivity.

In vivo imaging of nude mice with xenografts

Mice were anaesthetized using 0.5% pentobarbital (i.p. 50 mg kg^{-1}), administered ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ (37 MBq, 100 μ l; pH, 7.0) and then placed in a supine position in the centre of the field of view of a Siemens e.cam^{duet} single photon emission CT (SPECT) scanner equipped with a pinhole collimator for imaging. Acquisition of static images (300 000 counts) of the animals in the anteposition was performed with zoom 1.78 and

energy window 140 KeV, 15%, and data were digitally stored in a 128×128 matrix. To determine an optimal time point for HER2 imaging, the five mice bearing SKOV-3 xenografts were imaged at 0.5, 1, 2, 4, 6, 12 and 24 h after injection. The ratio of radioactive counts in the tumour to that in the contralateral equivalent region (T/NT) was calculated by drawing regions of interest at each time point. In addition, five mice bearing MDA-MB-231 xenografts were imaged at the same time as SKOV-3 bearing mice as negative controls after the administration of ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$. Meanwhile, to evaluate the specificity of binding of the ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ with HER2 receptors *in vivo*, 5 animals were pre-injected with 500 μ g of non-labelled $Z_{\text{HER2:342}}$, to saturate the HER2 receptors of tumours, and imaged 4 h after the injection of the ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ molecular probe via the tail vein.

Statistical analysis

Variables were expressed as mean value plus standard deviation ($\bar{x} \pm \text{sd}$). Analysis of variance (ANOVA) or a non-parametric test on rank transformed data was used to analyse the variation of tumour uptake in the biodistribution experiment and ratios of T/NT between different time points in the same group. Variables between the SKOV-3 and MDA-MB-231 groups at the same time point were analysed using the Student's *t* test. A *p*-value < 0.05 was considered significant.

RESULTS

Peptide synthesis and characterization

$Z_{\text{HER2:342}}$ was successfully synthesized using Fmoc solid phase peptide synthesis, and the chelator Gly-(D)-Ala-Gly-Gly was introduced at the N-terminus of $Z_{\text{HER2:342}}$. Results from RP-HPLC analysis of the synthesized compound Gly-(D)-Ala-Gly-Gly- $Z_{\text{HER2:342}}$ are shown in Figure 2a. The molecular weight of the synthesized compound as determined by mass spectrometry was 7.047 kDa, which correlated well with the theoretical mass.

Radiolabelling of $Z_{\text{HER2:342}}$

The labelling efficiency was $98.10 \pm 1.73\%$ ($n = 5$), and a high radioactive purity of the labelled conjugates with $95.57 \pm 0.54\%$ ($n = 5$) was obtained, which was sufficient to use for imaging without further purification. RP-HPLC analysis showed that the retention time of ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ was about 18.7 min (Figure 2b), whereas that of the free ^{99m}Tc was about 5 min (Figure 2c).

In vitro stability analysis

Retention of stability is an important characteristic for radio-labelled molecular probes. The initial radiochemical purity of the labelled conjugates was determined to be $98.10 \pm 1.73\%$ ($n = 5$), and it remained at a high level over a 6 h period. Even at 24 h, the radiochemical purity was $85\% \pm 0.53\%$ ($n = 5$), indicating that the radiolabelled conjugate had no significant degradation and off-labelling over time. Moreover, the RP-HPLC analysis showed that the radiolabelled molecular probe remained highly stable both in saline and in fresh human serum over time (Figure 3). When the ^{99m}Tc -labelled molecular probe was incubated in saline or fresh human serum at 37 °C, no significant difference in radiochemical purity was observed. The results showed that ^{99m}Tc -peptide- $Z_{\text{HER2:342}}$ was stable against degradation by blood plasma

proteases and was tolerant in human serum at 37°C, which mimicked conditions *in vivo*.

Biodistribution of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$

Biodistribution patterns of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ in mice bearing SKOV-3 xenografts are summarized in Figure 4a. Other than at tumour sites, radioactive accumulation of labelled conjugates was found primarily in the kidneys. The radioactive accumulation in SKOV-3 xenografts was higher than that for organs other than the kidneys. The tumour uptake was 4.611 ± 1.330 , 7.717 ± 0.925 , 8.218 ± 1.410 and $7.883 \pm 1.034\%$ ID/g at 1, 4, 6 and 8 h, respectively. Tumour uptake of SKOV-3 xenografts at 4, 6 and 8 h was higher than that at 1 h ($p < 0.05$); while there was no significant difference between the 4-, 6- and 8-h time points ($p > 0.05$). The radioactivity levels measured in blood, heart, bone, lung and muscle were very low, showing that the radiochemical was rapidly cleared in non-targeted organs. Together, the high tumour uptake in SKOV-3 xenografts and

rapid clearance from blood and non-specific compartments provided high tumour-to-organ ratios (Table 1). Pre-injection with an excess of non-labelled conjugates decreased tumour uptake from 8.218 ± 1.410 to $0.97 \pm 0.215\%$ ID/g ($p < 0.05$) 4 h after injection of the labelled conjugates, indicating that the radioactive uptake in tumour was HER2 receptor-specific and that ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ had good tumour-targeting properties (Figure 5). The biodistribution of labelled conjugates in mice bearing MDA-MB-231 xenografts is shown in Figure 4b. Tumour uptake in SKOV-3 xenografts was higher than that of MDA-MB-231 xenografts at 1, 4, 6 and 8 h ($p < 0.05$), demonstrating good specificity and sensitivity of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ in the detection of HER2 receptors.

In vivo imaging of nude mice with xenografts

Tumours of SKOV-3 bearing mice were visualized as early as 30 min and were shown most clearly at 6 h after the administration of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$. The image contrast improved over time up to 6 h, and the tumour was still clearly imaged even after 24 h (Figure 6a). A high accumulation also was observed in the kidneys, which corresponded well with the biodistribution data. Radioactivity accumulation was not detected in the thyroid, which is generally known to take up free ^{99m}Tc -pertechnetate. Moreover, ratios of radioactive counts in tumours to those in the contralateral equivalent non-tumour regions (T/NT ratios) were 2.93 ± 0.75 , 4.50 ± 0.43 , 5.72 ± 1.40 , 7.88 ± 1.78 and 8.26 ± 1.56 at 30 min, 1, 2, 4 and 6 h, respectively. The ratio of T/NT was highest at 6 h, but there was no significant difference between 4 and 6 h. However, there was no obvious uptake in low HER2-expressing MDA-MB-231 xenografts on SPECT images at any time after the injection of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ (Figure 6b). The T/NT ratios were 1.25 ± 0.31 , 1.58 ± 0.39 , 1.99 ± 0.43 , 2.56 ± 1.46 , and 2.58 ± 0.50 at 30 min, 1, 2, 4 and 6 h, respectively. The high HER2-expressing SKOV-3 xenografts accumulated significantly more radioactivity than did the low HER2-expressing MDA-MB-231 xenografts, with significantly different T/NT ratios between the two types of xenografts at all time points ($p < 0.05$). The radioactive uptake in tumours was further confirmed to be HER2 receptor-specific, as the imaging could be blocked effectively by pre-saturation of receptors with unlabelled $\text{Z}_{\text{HER2:342}}$ (Figure 6c).

Figure 2. The synthesized compound, Gly-(D) Ala-Gly-Gly- $\text{Z}_{\text{HER2:342}}$ was analysed by reversed-phase high performance liquid chromatography (RP-HPLC) (a). RP-HPLC analysis of Technetium-99m (^{99m}Tc)-peptide- $\text{Z}_{\text{HER2:342}}$ (b) and free ^{99m}Tc (c) was also performed.

Figure 3. *In vitro* stability of Technetium-99m (^{99m}Tc)-peptide- $\text{Z}_{\text{HER2:342}}$.

Figure 4. Biodistribution (%ID/g) of Technetium-99m (^{99m}Tc)-peptide- $\text{Z}_{\text{HER2:342}}$ in BALB/c nude mice bearing SKOV-3 xenografts (a) and MDA-MB-231 xenografts (b). %ID/g is the percentage of injected dose per gram of tissue.

DISCUSSION

The HER2 receptor, which is targeted by $\text{Z}_{\text{HER2:342}}$, is overexpressed in a proportion of carcinomas, including those of the breast, ovary and urinary bladder. Various studies have detected

HER2 expression in the range of 1.8–76% of ovarian carcinomas,¹ but not in normal ovaries or benign ovarian tumours.¹⁸ HER2 expression is associated with the malignant tumour phenotype, which is more likely to recur and metastasize.¹⁹ Targeting of HER2-expressing tumours by the humanized monoclonal antibody trastuzumab prolongs patient survival,² thus the efficient detection of HER2 can provide important diagnostic information and greatly influence patient management.

Radionuclide molecular imaging enables the detection of HER2 *in vivo* by a non-invasive procedure in both primary tumours and metastases. Application of this technique can avoid the disadvantages of biopsy sampling errors, especially when discordance in HER2 status exists between the primary tumour and metastatic tissues. The Affibody molecule is a 58-amino acid and cysteine-free three-helix bundle protein and it is a new class of small targeting proteins. The major advantage of the Affibody molecule as a targeting agent is the rapid tumour penetration and blood clearance from non-specific compartments. Another advantage is that the tracer can be produced completely by chemical synthesis instead of recombinant technology, which enables the site-specific introduction of the chelator and reduces production costs by approximately one-half.

The Affibody molecule $\text{Z}_{\text{HER2:342}}$, which binds to HER2 with an apparent affinity of 22 pM, has demonstrated a clear advantage in tumour targeting when labelled by radioiodine.^{8,9} Other radioisotopes, such as ^{111}In , ^{68}Ga , ^{186}Re , ^{177}Lu and ^{18}F , also have been used to label Affibody molecules and for the imaging of HER2 expression in metastatic breast cancer.^{10–14} However, ^{99m}Tc is likely the most suitable nuclide for SPECT as a label, with many advantages over other isotopes, such as having appropriate gamma energy (140 KeV) for SPECT imaging, easy preparation and a good safety profile for use in basic and clinical fields. Chelators, such as mercaptoacetyltriglycyl (maGGG),¹⁶ mercaptoacetyl-triseryl (maSSS),²⁰ mercaptoacetyl-glutamyl-seryl-glutamyl (maESE),²¹ and mercaptoacetyl-Glu-Glu-Glu (maEEE)²² have been used for labelling $\text{Z}_{\text{HER2:342}}$ with ^{99m}Tc in earlier studies. However, they all have advantages and disadvantages, an optimal chelator is still not available. Recently, the chelator Gly-(D) Ala-Gly-Gly was successfully used in the

Table 1. Tumour-to-organ ratios of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ in BALB/c nude mice bearing SKOV-3 xenografts and MDA-MB-231 xenografts

Tumor-to-organ ratio	1 h	4 h	6 h	8 h
T/blood				
SKOV3	4.101 ± 0.592	9.932 ± 1.594	13.283 ± 4.273	13.390 ± 2.513
MDA-MB-231	0.122 ± 0.058	0.203 ± 0.118	0.574 ± 0.069	0.232 ± 0.250
T/liver				
SKOV3	2.501 ± 0.587	3.721 ± 0.819	4.116 ± 0.513	4.298 ± 0.566
MDA-MB-231	0.122 ± 0.058	0.099 ± 0.041	0.281 ± 0.130	1.393 ± 1.924
T/muscle				
SKOV3	11.786 ± 2.235	12.778 ± 2.597	15.427 ± 3.378	15.552 ± 1.900
MDA-MB-231	1.005 ± 0.242	1.852 ± 1.685	1.918 ± 0.931	0.058 ± 0.077

Figure 5. Specificity of Technetium-99m (^{99m}Tc)-peptide- $\text{Z}_{\text{HER2:342}}$ uptake in BALB/c nude mice bearing SKOV-3 xenografts 4 h post-injection. Data are presented as %ID/g. For HER2 receptor saturation, animals in the blocked group were intravenously injected with 500 μg of unlabelled $\text{Z}_{\text{HER2:342}}$ 1 h before the injection of labelled conjugates. %ID/g is the percentage of injected dose per gram of tissue.

labelling of antisense peptide nucleic acids,²³ but it has not been used in the labelling of Affibody molecules. In this study, ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ was synthesized using Gly-(D) Ala-

Gly-Gly as a chelator, and the biophysical characterization showed successful synthesis and labelling of $\text{Z}_{\text{HER2:342}}$ by ^{99m}Tc . The high labelling efficiency of >98% and radiochemical purity of >95% indicated that this labelling method is superior to other methods by its simple protocol and lack of requirement for further purification.^{16,20–22} The experiments in this study demonstrated that the introduction of chelator sequence, Gly-(D) Ala-Gly-Gly, into the N-terminus of $\text{Z}_{\text{HER2:342}}$ did not have a negative effect on the HER2-specific binding capacity, which was in agreement with previous reports.^{16,20–22} Incubation of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ with fresh human serum also did not significantly decrease its activity compared with incubation in saline, showing that the labelled conjugates were stable against degradation by blood plasma proteases.

The biodistribution studies indicated an adequate *in vivo* stability of the labelled conjugates. By imaging, there was no obvious uptake in salivary gland, thyroid and stomach, which are generally known to accumulate pertechnetate; these results indicated that free pertechnetate was not released *in vivo*. The favourable biodistribution pattern of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ provided high-contrast images in SKOV-3 xenografts. The molecular imaging showed an obvious tumour uptake of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$ in HER2-expressing SKOV-3 xenografts, which was significantly higher than that in MDA-MB-231 xenografts with low HER2 expression.

Specific targeting is an important consideration for the development of a radiolabelled conjugate for clinical use. In this study, pre-saturation by excess unlabelled conjugates caused

Figure 6. Single photon emission CT planar imaging with Technetium-99m (^{99m}Tc)-peptide- $\text{Z}_{\text{HER2:342}}$ in BALB/c nude mice bearing HER2-expressing SKOV-3 xenografts (a) showing the uptake of the tumour (arrow) and low HER2-expressing MDA-MB-231 xenografts (b) at 30 min and 1, 2, 4, 6, 12 and 24 h after the administration of ^{99m}Tc -peptide- $\text{Z}_{\text{HER2:342}}$. Tumours of SKOV-3 bearing mice were blocked with a pre-injection of excess unlabelled $\text{Z}_{\text{HER2:342}}$ to saturate HER2 receptors (c).

a significant decrease in tumour uptake in the *in vivo* biodistribution and imaging experiments, indicating that the uptake in tumour was HER2 receptor specific. Different excretion pathways and uptake in organs are also an important issue in the design of targeting conjugates. However, the shortage of knowledge concerning the factors that control the biodistribution of the molecular probe is a challenge to all of us. The use of maGGG as a chelator provides labelled conjugates with good tumour-targeting properties, but a high amount of these molecules are eliminated via hepatobiliary excretion.¹⁶ The hepatobiliary excretion creates problems with imaging targets in the abdomen because of an elevated background. Renal excretion is preferred over hepatobiliary excretion, as the well-defined shape of the kidneys in combination with SPECT/CT can reduce the incidence of image misinterpretation. Substitution of maGGG with maSSS in ^{99m}Tc-Z_{HER2:342} has been reported to cause a shift from hepatobiliary to renal clearance.²⁰ Use of the chelator maESE in the ^{99m}Tc-labelled Z_{HER2:342} has decreased both liver and renal accumulation, resulting in improved targeting and imaging in the abdominal area.²²

In this study, the predominant clearance mechanism for the ^{99m}Tc-peptide-Z_{HER2:342} molecular probe, using Gly-(D) Ala-Gly-Gly as a chelator, was via renal excretion, while its elimination from other non-targeted organs (*i.e.* intestine) was a minor pathway. However, the renal excretion mechanisms are not clear and should be studied further in future experiments. Relative to ¹²⁵I labelled Z_{HER2:342}, ^{99m}Tc-peptide-Z_{HER2:342} is a superior probe owing to a higher tumour uptake and reduced renal retention of the radioactivity.¹⁶ Compared with other chelators reported in the previous study, such as maSSS, maESE, maEEE or mercaptoacetyl-seryl-lysyl-seryl (maSKS), for labelling Z_{HER2:342} with ^{99m}Tc, the distribution of ^{99m}Tc-Gly-(D) Ala-Gly-Gly-Z_{HER2:}

₃₄₂ is favourable not only because of its markedly decreased retention in the kidneys and intestine but also because there is no negative effect on tumour uptake.^{20–22,24} Therefore, this molecular probe is a promising agent for the detection of HER2 expression *in vivo* with good tumour-targeting properties and favourable distribution.

Several limitations of this study may be addressed in future experiments. For instance, a direct comparison should be made to validate the superiority of this new radiotracer to other radiotracers reported previously. While the images obtained in this study with SPECT were sufficient to interpret the biodistribution of ^{99m}Tc-peptide-Z_{HER2:342}, a SPECT/CT scan should be performed to acquire clearer images. Given differences between human and rodent physiology, clinical studies ultimately would be needed to clarify the distribution and specificity of ^{99m}Tc-peptide-Z_{HER2:342} for HER2-expressing tumours in patients.

CONCLUSION

The ^{99m}Tc-peptide-Z_{HER2:342} molecular probe is a promising tracer agent that may be used for molecular imaging of HER2-expressing malignant tumours. The results in this study provide a foundation for the future development of protocols for earlier visual detection of cancer in the clinical setting.

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